"ISOLATION, IDENTIFICATION AND DRUG RESISTANCE TESTING OF GRAM-NEGATIVE BACILLI AT A TERTIARY-CARE HOSPITAL WITH SPECIAL EMPHASIS ON THE PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF CARBAPENEM RESISTANCE"

SYNOPSIS SUBMITTED

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1.0 BACKGROUND

The “antibiotic era” may soon come to an end because the initial and seemingly unstoppable success of antibiotics has been matched by an escalation of resistance mechanisms in bacteria **(1)**. The discovery of every new antibiotic has been quickly accompanied by the emergence of resistance bacteria **(2)**. Bacterial resistance is a constantly evolving survival tactic that has enabled bacteria to outlast available antibiotics. Even in the early days of antibiotics, penicillinase-producing *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) were documented soon after the introduction of penicillin and methicillin respectively **(3).** Although multidrug resistant gram-positive organisms, such as MRSA and vancomycin-resistant enterococci, have stolen media attention and led the pharmaceutical industry to focus efforts on developing novel antibiotics, a more disturbing development has been the simultaneous development of multidrug resistance among gram-negative bacilli. Gram-negative organisms still account for most nosocomial infections, including pneumonia, surgical-site infections, intra-abdominal sepsis, and urosepsis, and are re-emerging as a significant cause of bloodstream infections **(4)**.

Resistance among gram-negative bacilli is not a new phenomenon. The first report of penicillin-inactivating enzymes in *Escherichia coli* was presented soon after the introduction of the drug in the 1940s **(5)**. Organisms such as *Pseudomonas aeruginosa* have always had a remarkable ability to evade new antimicrobial therapies and develop resistance; however, where resistance did develop, at least some other therapeutic options were available for treatment. More recently, pan-resistant gram-negative bacilli have been documented in hospitals all over the world. Few new antibiotic classes, besides the glycylcyclines, have been developed in the last 20 years **(6)**, and this is of great concern in view of the appearance of multi-drug resistant strains, and even pan-drug resistant strains that are resistant to all available antimicrobial agents **(7)**.

Gram-negative bacilli can become resistant to antimicrobials by four general mechanisms:

i) Production of enzymes that inactivate the drug;

ii) Mutations at the binding site that prevent the concerned drug from binding;

iii) Down-regulation of outer-membrane proteins that prevent the drug from reaching the periplasmic space; and

iv) Efflux pumps that pump drugs efficiently out of the cytoplasm or the periplasmic space. Several resistance mechanisms may be at work at the same time to allow the multidrug-resistant phenotype to be expressed,

The production of β-lactamases is among the commonest and clinically most significant resistance mechanisms displayed by gram-negative bacilli. β-lactamases are enzymes that hydrolyze the β-lactam ring and inactivate beta-lactam antimicrobials. They are typically classified by either the Ambler or the Bush-Jacoby-Medeiros systems **(8, 9)**.

The Ambler system classifies β-lactamases into four classes based on amino acid sequence similarities. For example, enzymes in class A, C, and D are serine β-lactamases, whereas class B enzymes are zinc β-lactamases. The Bush-Jacoby-Medeiros classification divides β-lactamases into three (previously four) groups and multiple subgroups, based on substrate preference and inhibitor profiles.

Since their introduction to clinical practice, **carbapenems** have been among the most useful antibiotics for treating serious infections caused by Gram-negative nosocomial pathogens, including members of family Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* **(10).** Remarkable stability towards most β-lactamases, including the extended-spectrum β-lactamases (ESBLs) and AmpC-type β-lactamases, accounts for the remarkably broad spectrum of activity exhibited by carbapenems. Therefore, the emergence of β-lactamases with carbapenem-hydrolyzing activity (carbapenemases), is of major clinical

concern, especially because these drugs have been the most effective agents for the treatment of serious infections caused by ESBL-producing Enterobacteriaceae (**11,12**).

The geographic distribution of carbapenemase-producing Gram-negative bacilli remained limited until the mid-2000s, and was not comparable to the worldwide dissemination of ESBL-producing Enterobacteriaceae observed during the same period. More recently, however, the carbapenemases have undergone dramatic changes in terms of enzyme diversity, epidemiological patterns and distribution. The repertoire of acquired carbapenemases has become increasingly complex, including not only several different types of metallo-beta-lactamases (MBL) e. g., IMP, VIM, SPM, GIM, SIM, KHM, AIM, NDM and DIM) and allelic variants thereof, but also various types of class A (e.g. KPC, NMC/IMI and SME) and class D (e.g. OXA-23, OXA-24, OXA-48 and OXA-58) serine carbapenemases **(13)**. Strains producing some of these enzymes have spread widely in some places, attaining a high level of endemicity.

2.0 OBJECTIVES

The objectives of the study:

1. To identify carbapenem resistant Gram-negative bacilli isolated at Himalayan Hospital
2. To characterize their resistance to different antimicrobials
3. To characterize mechanisms of carbapenem resistance by phenotypic and genotypic methods

3.0 MATERIALS AND METHODS

3.1 General Methods

3.1.1. Samples - The study will include all the clinical specimen referred for bacteriological culture from outdoor (OPD) as well as Indoor (IPD) patients of all age groups and both sexes from different wards of tertiary care hospital. The specimen comprised of urine, blood, sputum, pus, CSF, body fluids, swabs, endotracheal aspirate, tracheal tube aspirate etc.

3.1.2 Sample size - The worldwide prevalence of carbapenem resistance in Gram-negative bacilli varies from 4 to 60%, depending on the epidemiological setting; in India, the average prevalence is 12% (Gupta et al., 2006). Assuming a prevalence level of 10% in our hospital on the basis of a recent pilot study, 2000 isolates were estimated at 5% level of significance with 0.05 effect size. Procurement of fresh specimens will be stopped once 200 carbapenem-resistant isolates were obtained, even if it means that fewer than 2000 isolates were screened.

3.2. Specimen processing

3.2.1. Isolation - Clinically significant specimens will be inoculated on MacConkey II Agar (BBL^{TM}) and other media as appropriate, and incubated at 37^0C for 18 - 24 hours.

3.2.2. Gram-staining – All colonies isolated in pure culture or in predominant culture from clinically significant specimens will be stained by Gram's Method. Gram-negative isolates will be then further tested for our study.

3.2.4. Identification of isolates - Carbapenem-resistant colonies of Gram-negative bacilli, as determined by Gram-staining will be then characterized biochemically and identified by standard methods (Lennette et al., 1985 & Forbes et al., 2007). After noting colony morphology and the presence or absence of swarming, isolates were tested for oxidase; Hugh-Leifson glucose; gas and H_2S production in triple sugar iron agar (TSI); fermentation

of sucrose, cellobiose, arabinose, xylose, and adonitol; O-Nitrophenyl-β-D-Galactopyranoside (ONPG) hydrolysis; Methyl red (MR); Voges-proskauer (VP); indole production; citrate utilization; urea hydrolysis; phenylalanine deamination; lysine and ornithine decarboxylation; arginine dihydrolase activity; motility; and pigment production. Isolates that defied identification by these standard methods, will be identified using the automated Vitek 2 system (bioMérieux).

3.2.3. Screening for carbapenem resistance – All Gram-negative isolates will be tested for carbapenem resistance by the disc diffusion and results will be interpreted according to the Clinical Laboratory Standard Institute (CLSI) guidelines (CLSI, 2013). The antimicrobials tested including ampicillin (10 mcg), amoxycillin-clavulanate (30 µg), sulbactam (10 µg), piperacillin-tazobactam (10 µg), cephalothin (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), aztreonam (30 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), cotrimoxazole (25 µg), tetracycline (30 µg), tigecycline (15 µg), chloramphenicol (30 µg), and colistin (10 µg).

Isolates which demonstrated a reduced susceptibility toward ertapenem, imipenem, or meropenem after upon preliminary testing with discs from HiMedia®, will be retested with Neo-Sensitabs™ from Rosco Diagnostica, Denmark for confirmation.

3.2.4. Sub-culturing and Preservation of Isolates- Gram negative bacterial isolates with confirmed resistance to carbapenems will be sub cultured and preserved for further studies. For short term storage of bacterial cultures, bacterial isolates will be sub cultured on MacConkey II Agar (BBL™) and maintained at $+4^0$C and sub-cultured monthly every 10 days. For long term storage, the isolates will be grown overnight on Nutrient agar, then

suspended in Brain Heart Infusion broth (HiMedia®) with 15% glycerol (Sigma-Aldrich®) in micro-centrifuge tubes and keep at -20^{0}C

The resistant isolates will be then characterized phenotypically and genotypically.

3.3. Characterization of carbapenemase resistance by phenotypic methods

3.3.1. Determination of minimum inhibitory concentration (MIC's) of carbapenem drugs

Isolates identified as carbapenem-resistant by the Kirby-Bauer method, will be further tested by the Epsilometer Test to determine minimal inhibitory concentrations (MICs) of the carbapenems: imipenem, meropenem and ertapenem. Testing will be done on BBL™ Mueller Hinton II Agar (cation adjusted) using Etest® strips (bioMérieux, France) with a range of 0.002 µg/ml to 32 µg/ml, according to the manufacturer's instructions. MIC results will be interpreted according to the CLSI guidelines (CLSI, 2013).

3.3.2. Modified Hodge test

Media used - BBL™ Mueller Hinton II Agar (Cation adjusted)

Antibiotic discs used - Neo-Sensitabs™ Ertapenem and Imipenem from Rosco Diagnostica, Denmark.

The test will be performed as follows-

- 0.5 McFarland dilution of *Escherichia coli* ATCC®25922™ will be prepared in 5 ml of saline.
- The above suspension will be diluted 1:10 by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of MHB or saline.

- The 1:10 dilution of *E.coli* ATCC 25922 will be swabbed on a Mueller Hinton II agar plate that will be allowed to dry for five minutes.
- A 10 µg ertapenem for fermenters and 10 µg imipenem for non-fermenters susceptibility disc will be placed in the centre of the test area.
- The test organism will be streaked in a straight line from the edge of the disc to the edge of the plate. Not more than four organisms will be tested on the same plate.
- Plates will be incubated at 36°C in ambient air for 18 hours.

Interpretation of Results*:*

After 18 hours of incubation, the plates will be examined for a clover-leaf shaped indentation of the zone of inhibition of *Escherichia coli* ATCC®25922™ around the streak of the test strain.

MHT Positive test will have a clover leaf-like indentation of the *E. coli* 25922 growing along the test organism growth streak within the disc diffusion zone.

MHT Negative test will have no growth of the *E. coli* 25922 along the test organism growth streak within the disc diffusion.

3.3.3. Carbapenemase detection and identification based on inhibitor profile.

The KPC and MBL Confirm Kit from Rosco Diagnostica, Denmark will be used for detection of *Klebsiella pneumoniae* carbapenemase (KPC), metallo-β-lactamases (MBL) and AmpC in Enterobacteriaceae. The test will be performed according to the manufacturer instructions. Briefly:

- Bacterial suspension of 0.5 McFarland will be lawn cultured on Mueller Hinton II agar (BBL™) medium by sterile swab stick.

- Then different tablets namely tablet A meropenem, tablet B meropenem and dipicolinic acid (MBL inhibitor), tablet C meropenem and cloxacillin (AmpC inhibitor), and tablet D meropenem and boronic acid (KPC inhibitor) will then placed on the surface of the medium.
- The plates will then incubated at 35^0C for 16-24 hours.

Interpretation of Results:

The zone of inhibition of tablet A will then compared to the zones of inhibition of each of the carbapenem-plus-inhibitor tablets (B, C, and D).

- If tablet B shows a zone difference of ≥5 mm from tablet A, the organism will be recorded as demonstrating MBL activity.
- If tablet D shows a zone difference of ≥5 mm from tablet A, the organism will be recorded as demonstrating KPC activity.
- If tablets C and D both show a zone difference of ≥5mm from tablet A, the organism will be recorded as demonstrating AmpC activity coupled with porin loss. (impermeability)

3.3.4. Rapid CARB Blue Kit

The above kit from Rosco Diagnostica, Denmark will be used for the carbapenemase detection in Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* spp. isolates

The test will be performed according to the manufacturer instructions. Briefly-

- Bacterial suspension of McFarland standard 2 will be prepared from Mueller Hinton II Agar (BBL™) plates.
- Fifty µL of this suspension will be mixed in 100 µL of 0.9 % saline solution at pH 5 in two separate tubes.

- The suspension will be vortexed for 1 minute and then maintained at room temperature for 30 minutes.
- The Imipenem(x2) +bromothymol blue and negative control tablet will then put into these solutions separately.
- The tubes will be incubated at 37 °C for 2 hours.

Interpretation of Results

- Green colour of the test suspension and blue colour of negative control will indicate a positive reaction.
- Yellow colour of the test suspension and green colour of the negative control also will indicate a positive reaction
- A light yellow colour of the negative control indicated an invalid test and an uninterpretable result.
- Blue or green colour of both the test suspension and the negative control indicated a negative result.

3.3.5. Neo-Rapid CARB Kit

This kit from Rosco Diagnostica, Denmark will be used for the detection of carbapenemase in Enterobacteriaceae and *Pseudomonas* spp. isolates

This test will be performed according to the manufacturer instructions. Briefly-

- Bacterial suspension of McFarland standard 4 will be prepared from Mueller Hinton II Agar (BBL^{TM}) plates.
- Fifty µL of this suspension will be mixed in 100 µL of 0.9 % saline solution at pH 7.5 in two separate tubes.

- The suspension will be vortexed for 1 minute and then maintained at room temperature for 30 minutes.
- The imipenem (x2) +indicator (CARB) and negative control tablet will then put into these solutions separately.
- The tubes will be incubated at 37 °C for 2 hours.

Interpretation of Results

- A change in the colour of the test suspension from red to orange or some shade of yellow indicated a positive reaction, provided the negative control remained red.
- Red colour of both test and negative control tubes indicated a negative reaction.

3.3.6 Carba NP test

This test will be used for the detection of carbapenemases in Enterobacteriaceae and *Pseudomonas* spp (Nordmann et al., 2012 d)

The test will be performed as follows-

- One calibrated loop (10 µL) of the tested strain directly recovered from the antibiogram will be resuspended in a 100 µl lysis buffer (PUReGeNe™).
- The above suspension will be vortexed for 1 minute and further incubated at room temperature for 30 minutes.
- 50 µL of the suspension, corresponding to the enzymatic bacterial suspension, will be mixed in a 96-well tray with 100 µL of solution made of 6 mg imipenem monohydrate (Sigma-Aldrich®) pH 7.8, phenol red solution, and 0.1 mmol/L ZnSO4.
- The phenol red solution will be prepared by mixing 2 mL of a phenol red (Sigma-Aldrich®) solution 0.5% (wt/vol) with 16.6 mL of distilled water. The pH value will then adjusted to 7.8 by adding drops of 1 N sodium hydroxide (NaOH).

- A microtiter plate with mixture of the phenol red solution and the enzymatic suspension being tested will be incubated at 37°C for a maximum of 2 hours.

Interpretation of Results

(I) If the colour of the well containing phenol red plus imipenem plus $ZnSO_4$ turned from red to yellow-orange but the well containing phenol red plus $ZnSO_4$ plus remained red, the strain produced a carbapenemase.

(II) If the colour of both the wells remained red, the strain will not a carbapenemase producer; and

(III) If both the well colour turned from red to yellow-orange, the test will be considered as not interpretable.

3.3.7. CarbAcineto NP Test .

The CarbAcineto NP test will be used for the detection of carbapenemase-producing *Acinetobacter* spp. (Dortet et al., 2014).

In this updated version, the lysis buffer will be replaced by a 5 M sodium chloride solution, avoiding any buffer effect, and the bacterial inoculum will be doubled from one-third to one-half of a calibrated loop (10 µl) to a full calibrated loop in order to increase the enzyme quantity.

- Briefly, a full calibrated loop (10 µl) of the tested strain will be recovered from MHA plates and resuspended in two 1.5-ml Eppendorf tubes/microtiter plates (A and B) containing 100 µl of 5 M NaCl.
- 50 µL of the bacterial suspension will be mixed in two separate well of a microtiter plate with one well containing 100 µL of phenol red plus 0.1 mmol/L ZnSO4 solution and other well containing 6 mg imipenem monohydrate (Sigma-Aldrich®) plus phenol

red plus 0.1 mmol/L ZnSO4. The microtiter plate will then incubated at 37°C for a maximum of 2 hours.

- Optical reading of the colour change of each well will be performed. The carbapenemase activity will be detected by a colour change of phenol red solution (red to yellow/orange) resulting from the hydrolysis of imipenem into a carboxylic derivative, leading to a decrease of the pH value.

Interpretation of Results

(i) If the colour of the well containing phenol red plus imipenem plus $ZnSO_4$ turned from red to yellow-orange but the well containing phenol red plus $ZnSO_4$ plus remained red, the strain produced a carbapenemase.

(ii) If the colour of both the wells remained red, the strain will not a carbapenemase producer; and

(iii) If both the well colour turned from red to yellow-orange, the test will considered as not interpretable

The 5 M NaCl solution was prepared from a dilution of NaCl powder (HiMedia™) in distilled water

3.3.8. Blue Carba Test

This test will be used for the detection of carbapenemases in Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* isolates (Pires et al., 2013).

The test will be performed as follows-

- The test solution consist of an aqueous solution of bromothymol blue (Fluka®) at 0.04% adjusted to pH 6.0, 0.1 mmol/liter ZnSO4, and 6 mg/ml of imipenem, with a final pH of 7.0.

- A negative-control solution (0.04% bromothymol blue solution, pH 7.0) will be prepared to control the influence of bacterial components or products in the pH of the solution.
- A loop (approximately 5 µl) of a pure bacterial culture recovered from Mueller-Hinton II agar (BBL^{TM}) will directly suspended in 100 µl of both test and negative-control solutions in a 96-well microtiter plate and incubated at 37°C with agitation at 150 rpm for 2 hours.

Interpretation of Results

- If the colour of the well containing bromothymol blue and $ZnSO_4$ plus imipenem turned from blue to green or yellow, but the well containing bromothymol blue and $ZnSO_4$ plus remained blue, the strain produced a carbapenemase.
- If the colour of the well containing bromothymol blue and $ZnSO_4$ plus imipenem turned from green to yellow, but the well containing bromothymol blue and $ZnSO_4$ plus remained green, the strain produced a carbapenemase.
- If both the wells remained the same shade of blue or green colour, the strain produced no carbapenemase.
- If both wells changed colour from blue to green or from green to yellow, it indicated an invalid test and an uninterpretable test result.

3.3.9. Carbapenem Inactivation Method (CIM)

This method will be used for the detection of carbapenemases in Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* spp. isolates (Zwaluw et al., 2015) as follows:

- A suspension will be prepared by suspending loopful of bacterial culture from the Mueller Hinton II agar (BBL^{TM}) or blood agar plate in 400 µl of sterile distilled water.

- Subsequently, a meropenem (BD BBL™ Sensi-Disc™) will be immersed in the suspension.
- The above suspension will be incubated for minimum of two hours at 35^0 C.
- After incubation, the disc will be removed from the suspension using an inoculation loop.
- The discs will be placed on a Mueller-Hinton agar plate inoculated with a susceptible *Escherichia coli* ATCC®25922™ indicator strain.
- The above plates will then incubate overnight at 35^0 C.

Interpretation of Results

- If the bacterial isolate produced carbapenemase, the meropenem in the susceptibility disc was inactivated allowing uninhibited growth of the susceptible indicator strain.
- Discs incubated in suspensions that did not contain carbapenemases yielded a clear inhibition zone in the susceptible indicator strain.

Molecular Methods

3.4.1 Polymerase Chain Reaction (PCR) will be utilized to amplify specific nucleic acid sequences of all known carbapenemase-encoding genes, using specific primer pairs. DNA will be extracted from bacteria by suspending a single colony in 100 µL of distilled water (95^0C for 10 min) followed by centrifugation of the cell suspension. The entire DNA solution (2 µL) will be subjected to PCR in a 50 µL reaction mixture containing 1x PCR buffer (10 mMTris–HCl, pH 8.3, 50 mMKCl, 1.5 mM $MgCl_2$), 200 µM of each deoxynucleotide triphosphate, 1U of Taq polymerase and variable concentration of specific primers, Amplification will be carried out by initial denaturation at 94^0C for 10 minutes, followed by 30 cycles of (94^0C for 40 seconds, 60^0C for 40 seconds and 72^0C for 60 seconds), with a final

elongation step at 72^0C for seven minutes. Amplicons will be visualized after running the reaction mixture at 100 V for one hour on 2% agarose gel containing ethidium bromide. A 100-bp and 50 bp DNA ladder will be used as a size marker.

3.6. Efflux pump mechanism

The efflux pump in the isolates will be tested by the following two methods

3.9.1. Ethidium bromide cartwheel method

3.9.2. Using Reserpine as efflux pump inhibitor

3.6 Data analysis - Data will be analyzed using the the Microsoft Excel or other statistical software

4.0 Major findings

- Carbapenem resistance rate is very high (11.9%).
- Majority of resistance seen in Intensive Care Unit (ICU).
- Maximum carbapenem resistance seen in non-fermenters.
- All resistant isolates carried blaNDM-1; some also carried blaVIM and blaOXA-48.
- Most blaVIM positive strains (83.3%) were *Pseudomonas aeruginosa*.
- All blaOXA-48 positive strains were *Klebsiella pneumoniae*
- blaKPC not detected in any of 184 carbapenem-resistant isolates.
- No phenotypic test for carbapenemases had >90% sensitivity.
- Efflux pump activity contributed to carbapenem resistance in 10.9% of tested isolates.
- Tigecycline and colistin remain the only alternative treatment options for the majority of carbapenem-resistant isolates.

5.0 Significance and Implications

The universal presence of blaNDM-1 in carbapenem-resistant Gram-negative bacilli in our study indicates an urgent need to develop clinically useful inhibitors of Class B beta-lactamases.

The additional contribution of efflux pumps to carbapenem resistance highlights the need for clinically useful inhibitors of prokaryotic efflux systems, which are promising targets of antimicrobial chemotherapy because a single efflux pump can confer resistance to multiple antimicrobial classes.

The wide variety of carbapenem resistance mechanisms in different geographical locations at different points in time stresses the need for ongoing surveillance to enable empirical therapy while waiting for sensitivity testing results.

The fact that existing phenotypic tests for detecting and characterizing carbapenemases suffer from suboptimal sensitivity (≤90%) emphasizes the need to improve on existing tests and develop new ones.

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